Effects of osmotic perturbation on $[Ca^{2+}]_i$ and pH_i in rabbit proximal tubular cells in primary culture

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Raat, N. J. H., C. H. van Os, and R. J. M. Bindels. Effects of osmotic perturbation on $[Ca^{2+}]_i$ and pH_i in rabbit proximal tubular cells in primary culture. *Am. J. Physiol.* 269 (*Renal Fluid Electrolyte Physiol.* 38): F205–F211, 1995.—The effects of anisosmotic media on intracellular Ca^{2+} and H^+ concentrations ($[Ca^{2+}]_i$ and pH_i, respectively) were studied to investigate whether these changes play a role in epithelial cell volume regulation. $[Ca^{2+}]_i$ and pH_i were measured in rabbit proximal tubular cells in primary culture using the fluorescent ratio probes fura 2 and 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein. Changing medium osmolarity from 300 to 150 mosmol resulted in a rapid transient increase in fura 2 ratio from 0.89 ± 0.02 to 1.15 ± 0.03, which lasted for several minutes and returned to base line within 10 min. The source of Ca^{2+} was extracellular as well as intracellular. Simultaneous with this increase in $[Ca^{2+}]_i$, cells slowly acidified from pH_i of 7.51 ± 0.03 to 6.86 ± 0.02. This osmotic swelling-induced acidification could not be explained by a decrease in the rate of Na^+/H^+ exchange or increase in the rate of Cl^-/HCO_3^- exchange. Subsequently increasing medium osmolarity from 150 to 500 mosmol decreased the fura 2 ratio below the initial level observed in isotonic media, while pH_i increased from 6.96 ± 0.02 to 7.37 ± 0.03. This decrease in $[Ca^{2+}]_i$ was due to inhibition of Ca^{2+} influx and to an increase in Ca^{2+} efflux. The osmotic shrinkage-induced alkalinization was slightly inhibited by ethylisopropylamiloride, indicative of activation of Na^+/H^+ exchange. To test whether an increase in $[Ca^{2+}]_i$ causes a decrease in pH_i or vice versa, pH_i and $[Ca^{2+}]_i$ were manipulated at isotonic conditions. Surprisingly, a decrease in $[Ca^{2+}]_i$ was accompanied by a decrease in pH_i, and an increase in pH_i resulted in an increase in $[Ca^{2+}]_i$, in the absence of osmotic perturbation. In conclusion, changes in $[Ca^{2+}]_i$ and pH_i, resulting from osmotic perturbation of proximal tubular cells in primary culture, appear to be independent phenomena. This study suggests that both $[Ca^{2+}]_i$ and pH_i play a role as second messengers in cell volume regulation.

intracellular calcium; intracellular pH; cell swelling; cell shrinking

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MOST CELLS EXPOSED to anisosmotic solutions respond by activating volume recovery systems to prevent cell damage by either cell swelling or cell shrinkage (6, 10, 14, 26). In addition, in epithelial tissues such as the proximal tubule (PT), which are not subjected to large changes in extracellular osmolarity, volume recovery systems will be needed when the rate of transcellular solute transport is suddenly altered. The rate of water and ion transport across the PT cell is the highest observed among epithelial tissues, and up to four times its intracellular volume can be transported each minute by an individual PT cell (17). Changes in the availability of transportable solutes or the presence of transport-modulating hormones will affect transport rate and can lead to imbalance of influx and efflux followed by cell volume perturbation.

An increase in intracellular calcium concentration ($[Ca^{2+}]_i$) could play a role in the process of regulatory volume decrease (RVD) observed after osmotic cell swelling (19). Such an increase in $[Ca^{2+}]_i$ could activate K^+ and Cl^- channels, which results in KCl efflux followed by water and restoration of cell volume. In PT cells of the rabbit, an increase in $[Ca^{2+}]_i$ after cell swelling has been reported (3, 18, 19, 31). In contrast to the multitude of studies dealing with osmotic swelling, few reports on cell shrinkage or regulatory volume increase (RVI) and $[Ca^{2+}]_i$ are studied. Because changes in $[Ca^{2+}]_i$ and intracellular pH (pH_i) are often interrelated, we studied both $[Ca^{2+}]_i$ and pH_i during osmotic perturbation of PT cells in primary culture.

MATERIALS AND METHODS

Materials. All chemicals were obtained from Merck (Darmstadt, Germany), unless otherwise specified. Bumetanide was obtained from Leo Pharmaceutical (Ballerup, Denmark). Fetal calf serum was purchased from Sera-Lab (Sussex, UK), and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and tris(hydroxymethyl)aminomethane (Tris) were from Research Organics (Cleveland, OH). Gentamicin was obtained from Schering (Kenilworth, NJ). Fura 2-acetoxymethyl ester (AM), 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein-AM (BCECF-AM), 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-AM (BAPTA-AM), and ethylisopropylamiloride (EIPA) were from Molecular Probes (Eugene, OR). The disodium salt of 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) was purchased from Sigma (St. Louis, MO).

Solutions and media. The isotonic (300 mosmol) medium was a Krebs-Henseleit buffer (KHB), which contained (in mM) 110 NaCl, 5 KCl, 2 NaH_2PO_4, 1.2 $MgSO_4$, 10 sodium acetate, 4 L-lactate, 10 D-glucose, 1 L-alanine, 1 $CaCl_2$, and 20 HEPES, calibrated with Tris to pH 7.4. Hypotonic medium (150 mosmol) was similar to this solution except that NaCl concentration was reduced to 50 mM. Hypertonic medium (500 mosmol) was isotonic KHB, to which 200 mM mannitol had been added. Nominally Ca^{2+}-free medium contained no $CaCl_2$, and 0.1 mM $LaCl_3$ was used to inhibit residual Ca^{2+} influx. Chloride-free medium was KHB in which NaCl and KCl had been replaced by Na^+ and K^+ gluconate. NH_4Cl medium was KHB in which 30 mM NaCl was replaced by NH_4Cl. The osmolarity of the solutions was checked with an osmometer (Osmette A; Precision Systems, Sudbury, MA) and adjusted to the desired value with mannitol. K_1 medium consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium (Imperial, Hampshire, UK) and Ham's F-12 medium (GIBCO, Paisley, UK), supplemented with gentamicin (10 μg/ml), $NaHCO_3$ (25 mM), glutamine (14 mM), 0.5% (vol/vol) 100× nonessential amino acids (GIBCO, Paisley, UK), insulin (5 μg/ml), transferrin (5 μg/ml), hydrocortisone (50 nM), prostaglandin E_1 (70 ng/ml), triiodothyronine (5 pM), and Na_2SeO_3 (50 nM), and pH was set at 7.4.

Primary culture of rabbit kidney PT cells. Rabbit kidney PT cells were isolated and subsequently cultured as described previously (24). Briefly, PT cells were immunodissected from rabbit kidney with monoclonal antibodies 85C8 and 101E12.

Cells were seeded at a density of 2×10^5 cells/cm^2 on round 22-mm coverslips coated with rat tail collagen ($\sim$40 μg/cm^2) and cultured to confluency in K_1 medium in an atmosphere equilibrated with 5% CO_2-95% air at 37°C. During the first 24 h in culture, K_1 was supplemented with 5% (vol/vol) fetal calf serum. Medium was changed every other day and the day before experiments were performed. PT cells were used 6 days after seeding.

Measurement of $[Ca^{2+}]_i$ and pH_i. PT cells cultured on round 22-mm coverslips were loaded with fura 2-AM for 1 h in KHB medium (300 mosmol) containing 5 μM fura 2-AM, 0.02% (wt/vol) Pluronic F-127, and 3 mM probenecid in a shaking water bath at 37°C. After being loaded, the coverslip with cells was transferred to a thermostatic "Leiden" chamber (12). The volume of the chamber was reduced to 200 μl by a Perspex insert. For experiments in which $[Ca^{2+}]_i$ and pH_i were measured simultaneously, cells were subsequently loaded in $\sim$200 μl KHB medium containing 1 μM BCECF-AM, until fluorescence intensity equaled that of fura 2 (usually within 5 min). The chamber was mounted on the stage of a Nikon Diaphot inverted microscope equipped with a 40× quartz oil-immersion objective to monitor single PT cells. Fluorescence was captured by a low-level charge-coupled device camera and further processed by TARDIS software on the Magi-Cal system (Applied Imaging, Tyne and Wear, UK). The Magi-Cal system has been extensively described by Neylon et al. (22). Cells were superfused with KHB at 37°C at a rate of 1.5 ml/min. Probenecid (0.3 mM) was present in the media to reduce dye leakage from the cells. Before experiments, cells were preincubated for 10 min in isotonic KHB. pH_i was calculated from the 490/440 nm ratio ($R_{490/440}$) of BCECF after calibration in a medium containing (in mM) 112 K^+ gluconate, 28 KCl, 10 NaCl, 1 $MgCl_2$, 0.01 $CaCl_2$, 5 HEPES, 10 D-glucose, 20 mannitol, and 0.01 nigericin. In this buffer, a four-point calibration series between pH 6.0 and 7.8 was prepared by adjusting pH with Tris. Calibration in eight single cells per coverslip from three different preparations were pooled, and a mean calibration curve was calculated. The data points were fitted by linear regression to obtain a correlation between $R_{490/440}$ and pH_i. Calibration of fura 2 ratios in situ were severely hindered by the effects of ionomycin and ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) on cultured PT cells. In most attempts, addition of ionomycin or EGTA resulted in detachment of cells from the coverslip or lysis of cells, observations that have been previously encountered and described by Rose et al. (24). Therefore, $[Ca^{2+}]_i$ levels are represented as fura 2 ratios.

Protocol for study of changes in $[Ca^{2+}]_i$ and pH_i after cell swelling and shrinkage. After being loaded with fluorescent probes, the cells were preincubated for 10 min in 300 mosmol KHB at 37°C, and, subsequently, $[Ca^{2+}]_i$ or pH_i were measured. For the first 5 min, cells were superfused with 300 mosmol of medium to obtain the initial level of fluorescence. To induce cell swelling, cells were superfused for 10 min with a 150 mosmol medium. After this period, cells were superfused for 10 min with a 500 mosmol medium. When the effect of EIPA on pH_i was tested during incubation in 500 mosmol, EIPA (10 μM) was added 5 min before incubation in hypertonic medium. After a NH_4Cl pulse, the rate of increase in pH_i was determined as described by Abrahamse et al. (1). The data were fitted to the following exponential form: $R_t = R_{st} - (R_{st} - R_{init}) \cdot e^{-kt}$. R_t is the $R_{490/440}$ at time t, R_{st} is the $R_{490/440}$ at steady state, R_{init} is the $R_{490/440}$ at $t = 0$, and k is the rate constant for pH_i recovery. The rate of the change in $R_{490/440}$ [dR/dt at pH_i 6.8 ($R_{6.8}$)] was calculated, as described by Simchowitz and Roos (27), as $dR/dt = k \cdot (R_{st} - R_{6.8})$. The rate of pH_i recovery (dpH_i/dt) was calculated from dR/dt using the calibration curve.

Statistics. Measurement were performed on cells derived from two or three different preparations. From each preparation 16 cells were measured. Statistical significance was determined by one-way analysis of variance or a paired t-test. Data are presented as means ± SE.

RESULTS

Effect of cell swelling on $[Ca^{2+}]_i$ and pH_i. Exposure of primary cultured PT cells to hypotonic medium resulted in a fast but transient increase in fura 2 ratio within the first minute (Fig. 1 and Table 1). Within 10 min, the fura 2 ratio had decreased below the initial level to 0.74 ± 0.01. A heterogeneity in the fura 2 ratio response was observed, even among cells measured on the same coverslip (Fig. 1, *A-C*). The various responses in fura 2

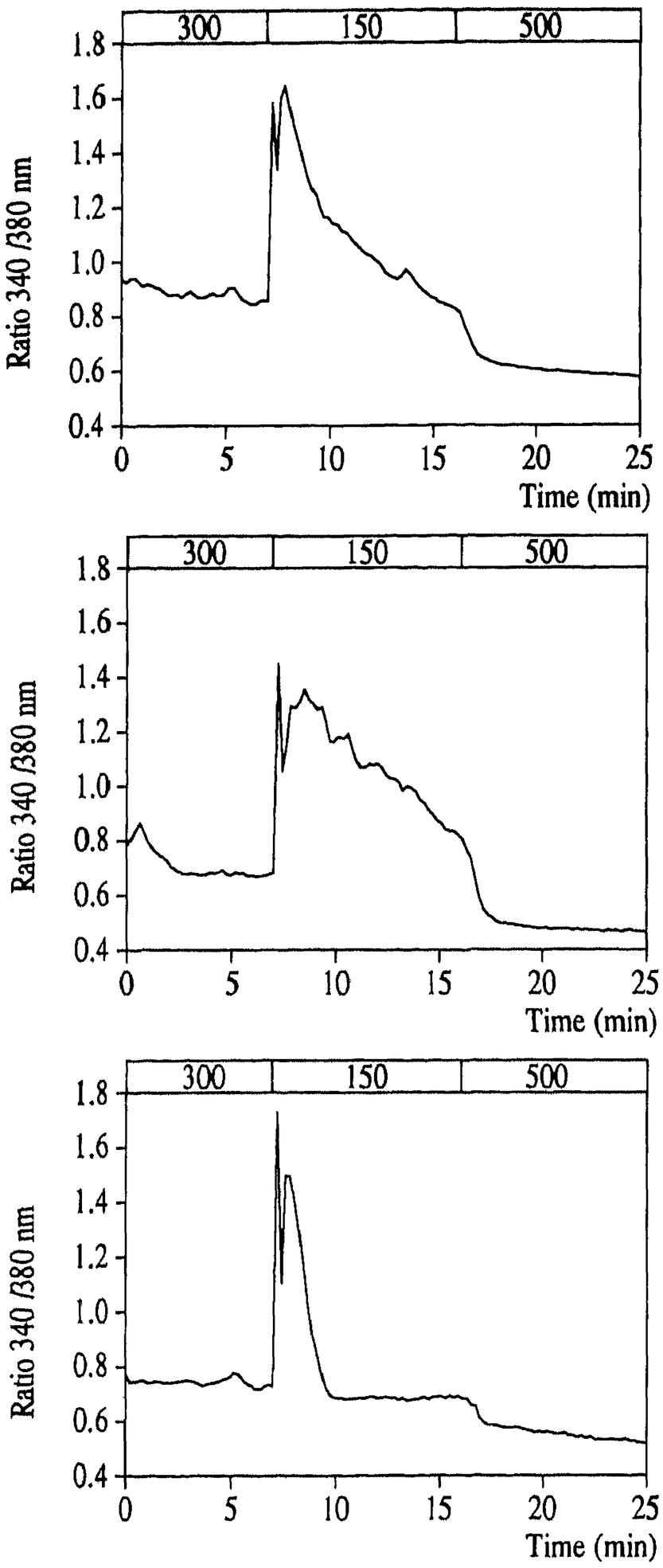

Fig. 1. Heterogeneity in cellular Ca^{2+} response in 3 individual primary cultured rabbit proximal tubule (PT) cells in same confluent monolayer during incubation in hypotonic (150 mosmol) and hypertonic (500 mosmol) media.

Table 1. *Change in $[Ca^{2+}]_i$ during cell swelling and subsequent cell shrinkage*

Condition	Fura 2 Ratio				
	Hypotonicity		Hypertonicity		
	Before	After	Before	After	n
Control	0.89 ± 0.02	1.15 ± 0.03*	0.74 ± 0.01	0.65 ± 0.01*	96
Ca^{2+} free	0.70 ± 0.01	0.92 ± 0.03*	0.69 ± 0.02	0.58 ± 0.01*	48
Thapsigargin	0.85 ± 0.02	0.95 ± 0.04*	0.73 ± 0.02	0.64 ± 0.02*	32
Thapsigargin + Ca^{2+} free	1.11 ± 0.05	0.66 ± 0.03*	0.67 ± 0.03	0.58 ± 0.02*	32

Values are means ± SE of n different cells from 2 or 3 separate isolations. Fura 2 ratio was measured during cell swelling in 150 mosmol medium and subsequent cell shrinkage in 500 mosmol medium. Initial values were compared with values directly after hypo- or hypertonicity in which nominally Ca^{2+}-free medium (+0.1 mM $LaCl_3$), 1 μM thapsigargin, or both were present. $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration. *$P < 0.05$, significantly different from value before osmotic perturbation.

ratio after a hypotonic shock could be divided into three groups, differing in their time course of recovery. Based on this arbitrary classification, the response shown in Fig. 1*A* was observed in ~46% of all responses. The other responses were observed at frequencies of ~26 and ~24% for Fig. 1, *B* and *C*, respectively. About 4% of the cells did not respond with an increase in $[Ca^{2+}]_i$. Before osmotic perturbation, pH_i was 7.51 ± 0.03 (Table 2) and, during hypotonicity, pH_i decreased slowly to 6.96 ± 0.02 within 10 min (Fig. 2). In contrast to the $[Ca^{2+}]_i$, pH_i responded rather uniformly.

The transient increase in $[Ca^{2+}]_i$ on exposure to hypotonic medium could arise from Ca^{2+} influx, from release of intracellular Ca^{2+}, or from both. As shown in Fig. 3*A*, PT cells preincubated in nominally Ca^{2+}-free medium for 3 min still responded with a transient increase in $[Ca^{2+}]_i$ (Table 1) on changing to a hypotonic nominally Ca^{2+}-free medium. Depletion of intracellular Ca^{2+} stores by preincubation with thapsigargin before osmotic perturbation still resulted in an increase in fura 2 ratio compared with control values (Table 1 and Fig. 3*B*). Depletion of intracellular Ca^{2+} stores by thapsigargin and subsequent exposure to a nominally Ca^{2+}-free hypotonic medium completely abolished the increase in fura 2 ratio (Table 1 and Fig. 3*C*). In this situation, $[Ca^{2+}]_i$ decreased significantly below the initial value.

On cell swelling, an acidification of the cytosol was observed. Due to the low extracellular Na^+ concentration ($[Na^+]$) in the hypotonic medium, a reversed operation of the Na^+/H^+ exchanger could be responsible for this acidification. However, cell acidification after hypotonic swelling was not significantly reduced by the addition of EIPA (Table 2). Other processes responsible for acidification could be efflux of H^+ from intracellular organelles or activation of Cl^-/HCO_3^- exchange (20). Although HCO_3^- was not present in our incubation medium, it is feasible that OH^- efflux via the anion exchanger is the cause of cellular acidification. In Cl^--free media, cell swelling is still accompanied by acidification (Table 2). In addition, the presence of 0.1 mM DIDS during the hypotonic shock was also without effect (Table 2). The possibility of conductive efflux of OH^-, as described by Muallem et al. (20) in osteosarcoma cells, was excluded by incubation in a high-K^+ (50 mM) hypotonic medium. High K^+ will depolarize PT cells and thereby reduce the driving force for conductive OH^- efflux, but acidification was still observed (data not shown).

Effects of cell shrinkage on $[Ca^{2+}]_i$ and pH_i. Changing the osmolarity from 300 to 500 mosmol resulted in a decrease in fura 2 ratio from a basal level of 0.82 ± 0.04 to 0.67 ± 0.03 (Fig. 4*A*). During this maneuver, pH_i increased from 7.69 ± 0.03 to 7.85 ± 0.04 (Fig. 4*B*). Superfusion of PT cells with 500 mosmol after superfusion with 150 mosmol for 10 min also resulted in a decrease in the fura 2 ratio and an increase in pH_i (Table 2 and Fig. 2). The observed decline in $[Ca^{2+}]_i$ on cell

Table 2. *Change in pH_i during cell swelling and subsequent cell shrinkage*

Condition	pH_i			
	Hypotonicity		Hypertonicity	n
	Before	After		
Control	7.55 ± 0.03	6.99 ± 0.02*	7.41 ± 0.03*	80
Cl^- free	7.74 ± 0.03	7.02 ± 0.03*	7.47 ± 0.04*	48
+EIPA	7.72 ± 0.05	7.03 ± 0.04*	7.49 ± 0.04*	32
+DIDS	7.63 ± 0.02	7.01 ± 0.02*	7.48 ± 0.05*	48

Values are means ± SE of n different cells from 2 or 3 separate isolations. pH_i was measured using 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) during cell swelling in 150 mosmol medium and subsequent cell shrinkage in 500 mosmol medium. Initial values were compared with values after 10 min in Cl^--free medium or in medium in which 10 μM ethylisopropyl amiloride (EIPA) or 0.1 mM 4,4′-diisothiocyanostilbene-2,2′-sulfonic acid (DIDS) was present. *$P < 0.05$, significantly different from value before osmotic perturbation.

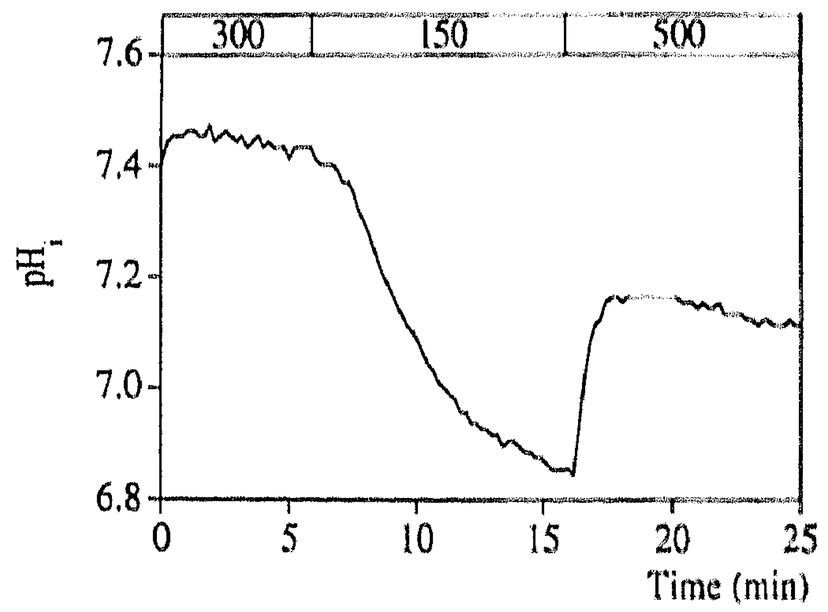

Fig. 2. Typical response in intracellular pH (pH_i) in an individual rabbit PT cell in a confluent monolayer after hypotonic (150 mosmol) and subsequent hypertonic incubation (500 mosmol). Homogenous response was observed in cells measured in 1 experiment or from different isolations.

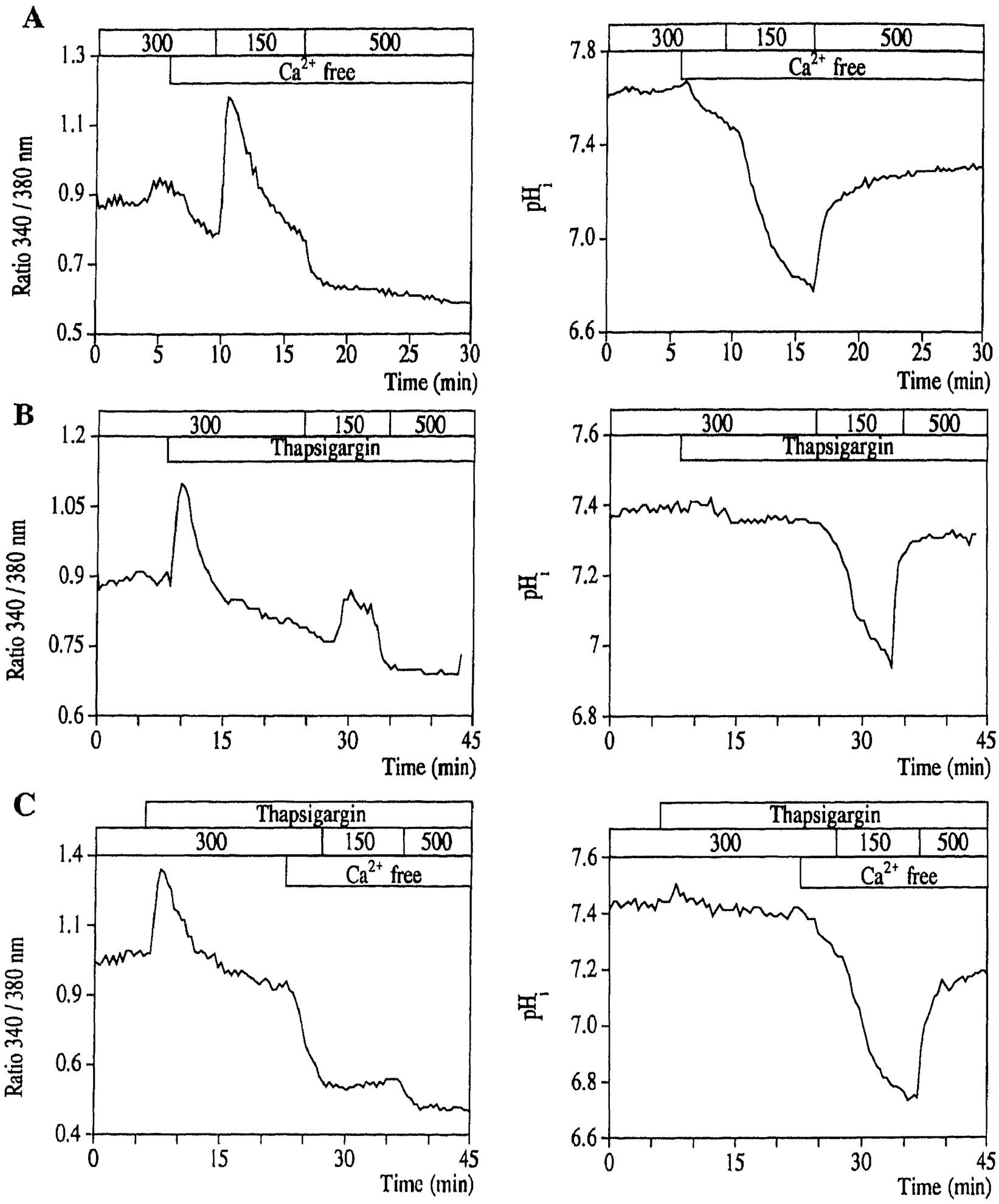

Fig. 3. *A*: typical response in simultaneously measured fura 2 ratio and pH_i in a single rabbit PT cell in a confluent monolayer during nominal Ca^{2+}-free hypo- and hypertonic incubation. During preincubation in nominally Ca^{2+}-free isotonic medium, a decrease in fura 2 ratio is observed, but an increase in fura 2 ratio is still present when cells are incubated in nominally Ca^{2+}-free hypotonic (150 mosmol) medium. Fura 2 ratio decreases when cells are subsequently placed in hypertonic (500 mosmol) medium. *B*: typical response in simultaneously measured fura 2 ratio and pH_i in a single rabbit PT cell in a confluent monolayer during hypo- and hypertonic incubation after depletion of intracellular Ca^{2+} stores by 1 μM thapsigargin. Addition of thapsigargin results in an increase in fura 2 ratio. After hypotonic (150 mosmol) incubation the rise in fura 2 ratio is significantly lowered compared with control. Fura 2 ratio decreases further when cells are subsequently placed in hypertonic (500 mosmol) medium. *C*: typical response in simultaneously measured fura 2 ratio and pH_i in a single rabbit PT cell in a confluent monolayer during hypo- and hypertonic incubation after initial depletion of intracellular Ca^{2+} stores by 1 μM thapsigargin and subsequent incubation in nominally Ca^{2+}-free hypotonic (150 mosmol) medium. Increase in fura 2 ratio is completely abolished. Fura 2 ratio further declines when cells are subsequently placed in hypertonic (500 mosmol) nominally Ca^{2+}-free medium.

shrinkage could be brought about by *1*) inhibition of Ca^{2+} influx not balanced by a decrease in Ca^{2+} efflux, *2*) increased Ca^{2+} sequestration by intracellular stores, or *3*) increased Ca^{2+} efflux. During incubation in nominally Ca^{2+}-free medium, the decline in [Ca^{2+}]$_i$ on cell shrinkage is still present (Fig. 3*A* and Table 1). The decrease in [Ca^{2+}]$_i$ is also observed in the presence of thapsigargin (Fig. 3*B*) and in the absence of Ca^{2+} and presence of thapsigargin (Fig. 3*C*). Because no specific inhibitors of the plasma membrane Ca^{2+}-ATPase are available at present, studies to directly test the involvement of this Ca^{2+} pump cannot be experimentally evaluated.

Whether Na^+/H^+ exchange is responsible for the alkalinization on cell shrinkage was tested using the inhibitor EIPA. Changing the medium osmolarity from 300 to 500 mosmol medium in the presence of EIPA did not reduce alkalinization. However, preswelling in 150 mosmol followed by 500 mosmol in the presence of EIPA resulted in a lower pH_i value than in the absence of EIPA (Table 2). When pH_i was acidified by an NH_4Cl pulse, the recovery of pH_i (dpH_i/dt) was 0.22 ± 0.05 pH units/min (means ± SE, n = 16), and this rate decreased significantly in the presence of EIPA to 0.07 ± 0.01 pH units/min (means ± SE, n = 12; $P < 0.05$).

Interdependence of [Ca^{2+}]$_i$ and pH_i. During cell swelling and shrinkage, changes in [Ca^{2+}]$_i$ and pH_i occur simultaneously, which raises the question of whether these changes are interrelated or independent. [Ca^{2+}]$_i$ increased in isotonic media after addition of thapsigargin, but this increase in [Ca^{2+}]$_i$ was not accompanied by a change in simultaneously measured pH_i (Fig. 3*A* and Table 3). Incubation of PT cells in isotonic nominally free Ca^{2+} medium reduced [Ca^{2+}]$_i$ and concomitantly decreased pH_i (Table 3). Incubation of PT cells in an isotonic medium at pH 8.5 raised pH_i and simultaneously increased [Ca^{2+}]$_i$ (Fig. 5*A* and Table 3). On the contrary, lowering the extracellular pH to 6.5 decreased pH_i as well as [Ca^{2+}]$_i$ (Fig. 5*B* and Table 3). Changes in

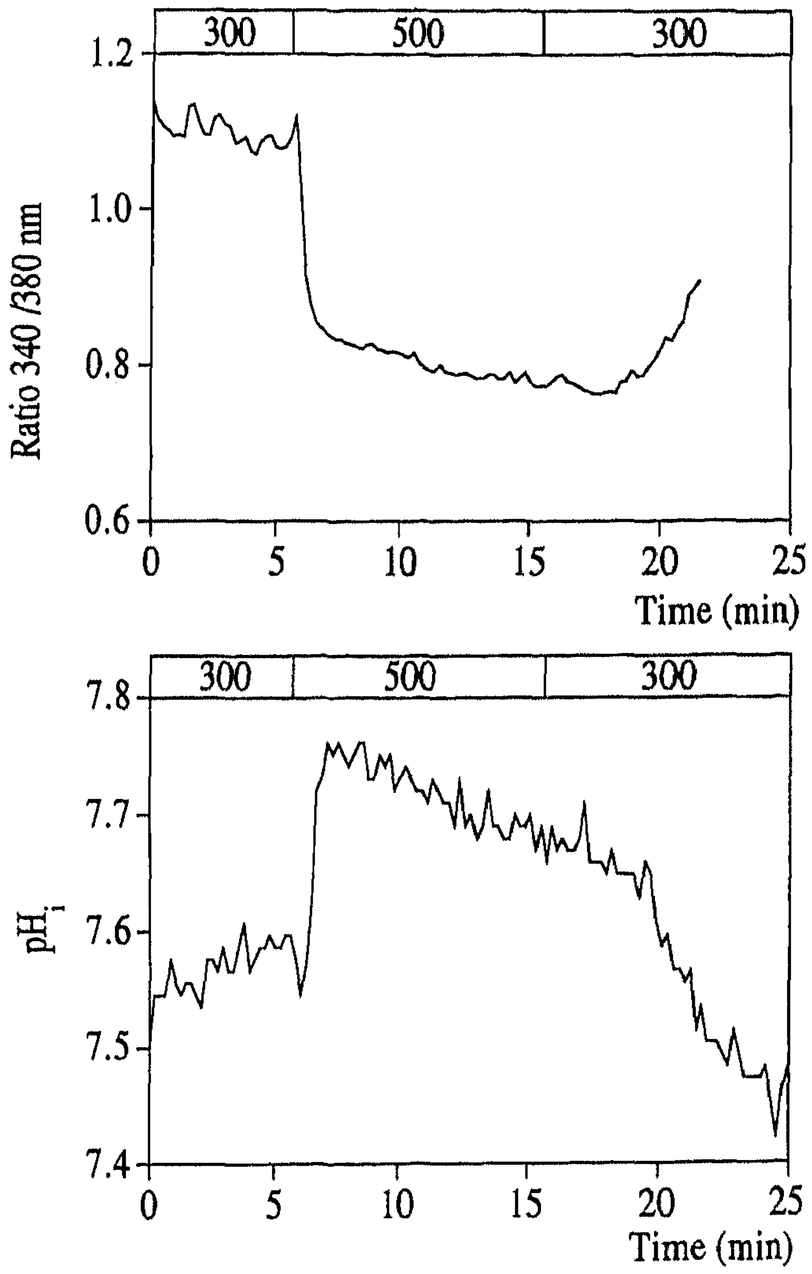

Fig. 4. *Top*: decrease in fura 2 ratio after hypertonic (500 mosmol) and subsequent isotonic incubation (300 mosmol) in a single cell in a confluent monolayer of rabbit PT cells. *Bottom*: increase in pH_i after hypertonic (500 mosmol) and subsequent isotonic incubation (300 mosmol) in 1 cell in a confluent monolayer of rabbit PT cells.

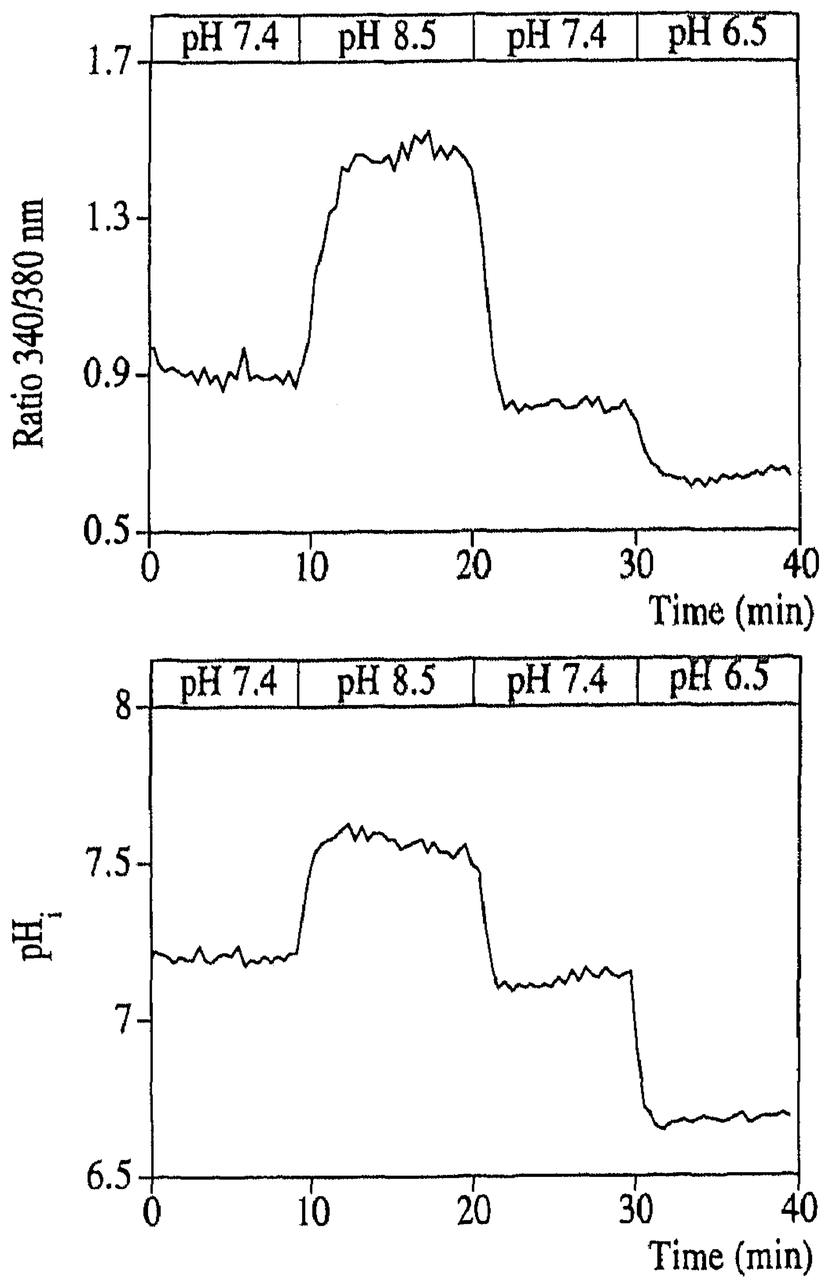

Fig. 5. *Top*: typical response in fura 2 ratio in a single cell in a confluent monolayer of rabbit PT cells after incubation in isotonic medium at pH values 8.5, 7.4, and 6.5. Fura 2 ratio becomes 1.41 ± 0.05, 0.84 ± 0.02, and 0.67 ± 0.02, respectively (means ± SE; $n = 32$). *Bottom*: typical response in pH_i measured in same cell as at *top* after incubation in isotonic medium at pH 8.5, 7.4, and 6.5. pH_i becomes 7.82 ± 0.06, 7.34 ± 0.05, and 6.84 ± 0.04, respectively (means ± SE; $n = 32$).

pH_i brought about by the NH_4Cl pulse method influenced [Ca^{2+}]$_i$ similar to that shown for extracellular pH changes (results not shown).

In addition, we used the Ca^{2+} chelator, BAPTA-AM, to buffer the Ca^{2+} peak generated by cell swelling. BAPTA already decreased resting [Ca^{2+}]$_i$ levels, since the fura 2 ratio declined from 0.88 ± 0.02 to 0.63 ± 0.02. This decline in [Ca^{2+}]$_i$ was accompanied by a decrease in pH_i from 7.29 ± 0.05 to 7.11 ± 0.05. However, cell acidification still occurred after cell swelling, despite the absence of the transient increase in [Ca^{2+}]$_i$ (Fig. 6, *A* and *B*). These results demonstrate clearly that, during cell volume perturbations, Ca^{2+} signals and pH_i can change independently.

Table 3. *Effect of pH_i perturbations on [Ca^{2+}]$_i$ and vice versa at isotonicity*

	Fura 2 Ratio		pH_i		
Condition	Before	After	Before	After	n
Thapsigargin	0.87 ± 0.02	1.14 ± 0.04*	7.32 ± 0.03	7.34 ± 0.03	32
Ca^{2+} free	0.89 ± 0.02	0.70 ± 0.03*	7.39 ± 0.03	7.27 ± 0.03*	48
pH_0 8.5	0.92 ± 0.02	1.41 ± 0.05*	7.37 ± 0.04	7.82 ± 0.06*	32
pH_0 6.5	0.84 ± 0.02	0.67 ± 0.02*	7.34 ± 0.05	6.84 ± 0.04*	32

Values are means ± SE of n different cells from 2 or 3 separate isolations. Fura 2 ratio and pH_i were measured simultaneously under isotonic conditions. Initial values were compared with values directly after conditions in which 1 μM thapsigargin, nominally Ca^{2+}-free medium (+0.1 mM $LaCl_3$), or Krebs-Henseleit buffer with high and low pH were present. *$P < 0.05$, significantly different from value before experimental manipulation.

DISCUSSION

In the present study, we observed, in PT cells in primary culture, abrupt changes in [Ca^{2+}]$_i$ and pH_i on osmotic perturbation. This raises the question of whether changes in [Ca^{2+}]$_i$ and pH_i are involved in activating the volume recovery mechanism or whether they result from transport processes activated by volume perturbation. After a hypotonic shock, [Ca^{2+}]$_i$ increased transiently in PT cells, which is in line with observations in other cell types (23). This rise in [Ca^{2+}]$_i$ has been postulated to start the process called RVD by activating K^+ and Cl^- channels (19). Also, in isolated perfused rabbit PT cells, [Ca^{2+}]$_i$ has been shown to play a role in RVD, although the [Ca^{2+}]$_i$ signal varies among different investigators (3, 4, 18, 31). In our study, no sustained Ca^{2+} level was observed as previously described by McCarty and O'Neil (18) and Beck et al. (3). Differences may be due to a variation in experimental conditions. We used cultured PT cells and measured [Ca^{2+}]$_i$ at 37°C, whereas Suzuki et al. (31), also using cultured PT cells, measured [Ca^{2+}]$_i$ at room temperature. In the other studies mentioned, isolated perfused tubules were used at 37°C. In contrast, McCarty and O'Neil (18) used

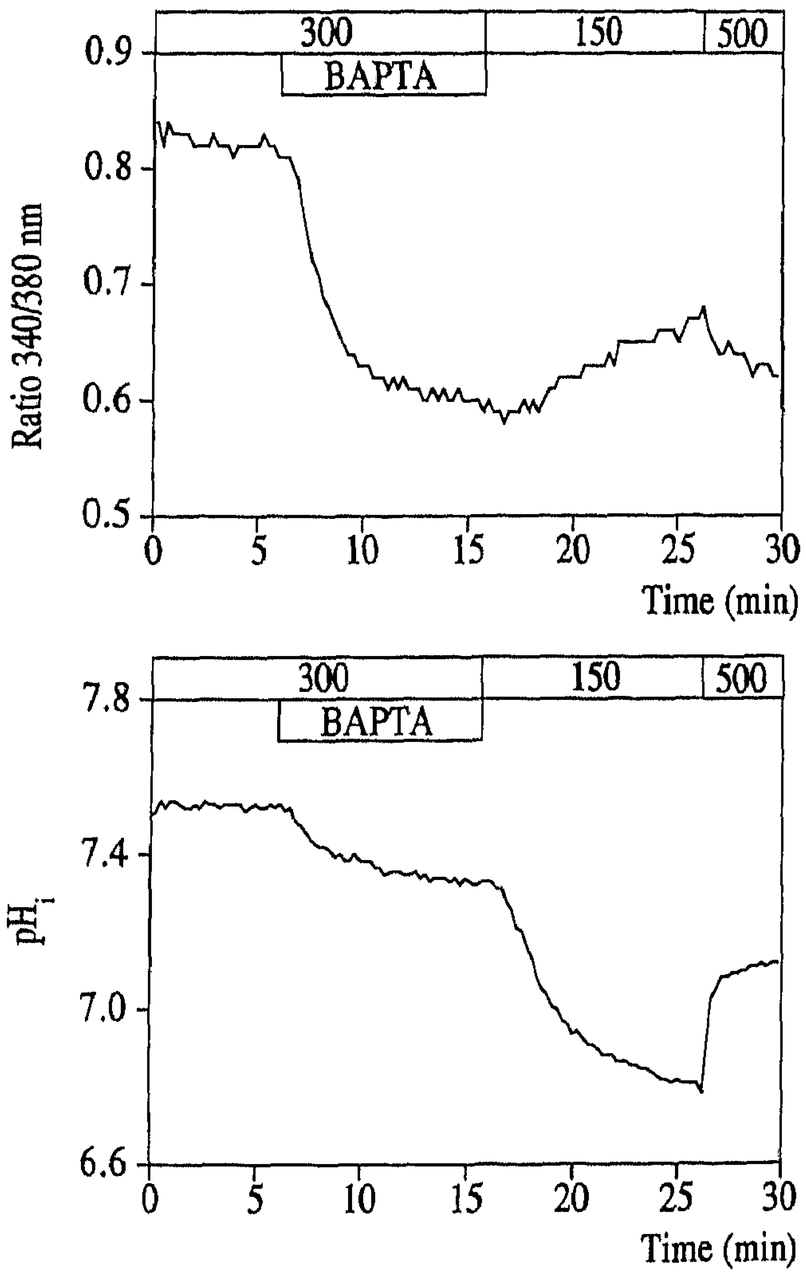

Fig. 6. *Top*: typical response in fura 2 ratio in a single rabbit PT cell in a confluent monolayer after loading for 10 min with 10 μM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester (BAPTA-AM). No significant increase in fura 2 ratio was observed when isotonic (300 mosmol) medium was changed for a hypotonic (150 mosmol) one. *Bottom*: typical response in pH_i measured in same cell as at *top* after loading for 10 min with 10 μM BAPTA-AM in isotonic (300 mosmol) medium. Acidification after hypotonic (150 mosmol) perturbation is still observed, despite buffering of Ca^{2+} spike.

proximal straight tubules instead of proximal convoluted tubules. Another variable has been the magnitude of the hypotonic shock, which ranged from 50 to 150 mosmol. In our study, the observed Ca^{2+} spike was only partially dependent on extracellular Ca^{2+}, in contrast to McCarty and O'Neil (18) and Breton et al. (4), who reported a complete dependence on extracellular Ca^{2+}. In the presence of thapsigargin, we still observed an increase in $[Ca^{2+}]_i$ after hypotonic incubation, but the height of the peak was substantially reduced. Our results demonstrate that, in cultured PT cells, osmotic swelling mobilizes calcium from extracellular as well as intracellular sources.

In addition to the rapid increase in $[Ca^{2+}]_i$, cultured PT cells also slowly acidified on cell swelling. Beck et al. (2) previously reported an acidification in perfused PTs, but, in the presence of HCO_3^-, a small alkalinization was observed. Intracellular acidification also occurred in osteosarcoma cells on cell swelling independent of the presence or absence of HCO_3^- (30). In addition, cell acidification after swelling was observed in human platelets (15) and Ehrlich cells (16). After dilution of the intracellular fluid by swelling, one would expect an alkalinization, suggesting that, in PT cells, additional systems could be involved. In search of mechanisms that could explain the swelling-induced cell acidification, we could exclude Na^+/H^+ exchange and Cl^-/HCO_3^- exchange. Livne and Hoffmann (15) suggested that increased metabolic acid production or K^+/H^+ exchange was responsible for the acidification. In Ehrlich cells, Cl^-/HCO_3^- exchange plays a role in acidification after cell swelling (16), whereas, in osteosarcoma cells, conductive efflux of OH^- was responsible (20). A conductive efflux could not be demonstrated in the present study, since depolarization did not affect acidification in cultured PT cells.

In contrast to osmotic swelling, osmotic shrinkage of cultured PT cells decreased $[Ca^{2+}]_i$. In nominally free Ca^{2+} medium, $[Ca^{2+}]_i$ decreased even further after a hypertonic shock, suggesting that an abrupt stop in Ca^{2+} influx is not the mechanism behind this Ca^{2+}-lowering event. Also, increased uptake of Ca^{2+} by the endothelial reticulum cannot explain the decline in $[Ca^{2+}]_i$, since thapsigargin did not prevent it. The only remaining possibility is an increased Ca^{2+} efflux, which is hard to prove experimentally, in view of lack of specific inhibitors of plasma membrane Ca^{2+}-ATPases.

There are few studies reporting effects of hypertonicity on $[Ca^{2+}]_i$. In several studies, however, it was shown that RVI is independent of $[Ca^{2+}]_i$ (7, 8, 11). In rat thymic lymphocytes, hypertonic activation of Na^+/H^+ exchange also caused a rise in $[Ca^{2+}]_i$, which was a secondary effect, since $[Ca^{2+}]_i$ did not increase in Na^+-free solutions. It is not very likely that a decrease in $[Ca^{2+}]_i$ serves as a signal for activation of volume recovery mechanisms, since, in general, only Ca^{2+} spikes activate cells. In gastric parietal cells, Negulescu et al. (21) observed a 30% decrease in $[Ca^{2+}]_i$ after cell shrinkage but solely when $[Ca^{2+}]_i$ was first elevated by pretreatment of carbachol. Here, cell shrinkage was shown to be a selective inhibitor of Ca^{2+} influx. In our study, the decrease in $[Ca^{2+}]_i$ occurred without prior elevation of $[Ca^{2+}]_i$ by hormones.

Cell shrinkage induced cytosolic alkalinization, and the most likely explanation would be activation of Na^+/H^+ exchange, since this transport has been shown to be activated by hypertonicity in a variety of cell types (10). Surprisingly, EIPA did not prevent shrinkage-induced alkalinization of cultured PT cells. However, EIPA did inhibit the recovery in pH_i after acidification by a NH_4Cl pulse. An explanation could be that an isoform of the Na^+/H^+ exchanger, i.e., NHE2 (25), which exhibits reduced amiloride sensitivity and is present in the apical membrane of polarized cells, is responsible for the shrinkage-induced alkalinization.

In cultured PT cells, changes in $[Ca^{2+}]_i$ and pH_i take place simultaneously on cell volume perturbation. The present study and other studies have shown that $[Ca^{2+}]_i$ and pH_i affect each other also in the absence of cell volume perturbations (5, 9, 28, 29, 32). The dependence of $[Ca^{2+}]_i$ on pH_i at isotonicity appears to be tissue and species specific, since a change in pH_i has been shown to result in either a decreased, increased, or unchanged $[Ca^{2+}]_i$ level (9, 28, 29). In vascular smooth muscle cells (28), rat lymphocytes (9), chick cardiac myocytes (13), and cultured PT cells in the present study, an increase in pH_i at isotonicity resulted in a rise in $[Ca^{2+}]_i$, while a decline in $[Ca^{2+}]_i$ was observed after lowering pH_i. In contrast, in PT cells and in inner medullary collecting

duct cells (29), a decrease in $[Ca^{2+}]_i$ at isotonicity resulted in an cytosolic acidification.

Despite this obligatory coupling of $[Ca^{2+}]_i$ and pH_i at isotonicity, we could completely dissociate the rise in $[Ca^{2+}]_i$ induced by cell swelling from cytosolic acidification by using BAPTA. This Ca^{2+} chelator was able to prevent the rise in $[Ca^{2+}]_i$ on osmotic swelling, but the acidification still occurred. In addition, at isotonicity, an increase in pH_i was accompanied by an increase in $[Ca^{2+}]_i$, while, on cell shrinkage, pH_i increased, and $[Ca^{2+}]_i$ decreased. Therefore, changes in $[Ca^{2+}]_i$ and pH_i induced by cell volume perturbation are clearly dissociated. In addition, the time course of cell acidification is slower than the transient increase in $[Ca^{2+}]_i$, and the increase in $[Ca^{2+}]_i$ was heterogeneous, in contrast to the rather uniform decrease in pH_i. These facts support uncoupled and independent phenomena. Our observations suggest that $[Ca^{2+}]_i$, as well as pH_i, plays a role in the signal transduction cascade activated by cell volume sensors.

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